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Thanks in advance,

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Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis

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In eukaryotes, homologs of the bacterial MutS and MutL proteins function in DNA mismatch repair and recombination pathways. The *mutL* homolog *MLH1* is required for nuclear mismatch repair. Previously, cytological analysis of *MLH1*-deficient mice has implied a role for Mlh1 in crossing-over during meiosis. Here we demonstrate that *Saccharomyces cerevisiae* diploids containing a deletion of *MLH1* have reduced crossing-over in addition to a deficiency in the repair of mismatched DNA during meiosis. Absence of either of the meiosis-specific mutS homologs Msh4 or Msh5 results in a similar reduction in crossing-over. Analysis of an *mlh1 msh4* double mutant suggests that both genes act in the same pathway to promote crossing-over. All genetic markers analyzed in *mlh1* mutants display elevated frequencies of non-Mendelian segregation. Most of these events are postmeiotic segregations that represent unrepaired heteroduplex. These data suggest that either restorational repair is frequent or heteroduplex tracts are shorter in wild-type cells. Comparison of *mlh1* segregation data with that of *pms1, msh2, msh3,* and *msh6* mutants show that the ability to promote crossing-over is unique to *MLH1*. Taken together these observations indicate that both crossing-over and gene conversion require MutS and MutL functions and that Mlh1 represents an overlap between these two pathways. Models of Mlh1 function are discussed.

[*Key Words:* MutL homologs; MutS homologs; mismatch repair; meiosis; crossing-over; gene conversion.]

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In eukaryotes, proteins homologous to the MutS and MutL components of the bacterial long-patch mismatch repair system have evolved to perform both DNA repair and recombination functions. In *Saccharomyces cerevisiae* the MutS homologs Msh2, Msh3, and Msh6 and the MutL homologs Pms1 and Mlh1 are involved in the repair of mismatched DNA formed during replication and recombination (Williamson et al. 1985; Kramer et al. 1989; Reenan and Kolodner 1992b; New et al. 1993; Prolla et al. 1994a; Johnson et al. 1996; Marsischky et al. 1996; for review, see Kolodner 1996; Crouse 1997). Several of these proteins also function to prevent inappropriate genetic exchanges between nonidentical or homeologous DNA sequences (Selva et al. 1995; Chambers et al. 1996; Datta et al. 1996; Hunter et al. 1996; N. Hunter and R. H. Borts unpubl.). Other MutS homologs possess recombination functions that appear to be unrelated to mismatch repair. The *S. cerevisiae* Msh4 and Msh5 proteins specifically promote crossing-over during meiosis and the Swi4 protein of *Schizosaccharomyces pombe* is thought to be involved in the termination of DNA synthesis during the copying of the mating-type cassette during switching (Fleck et al. 1992; Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995).

In this study we examine the activities of the mismatch repair system during meiosis in *S. cerevisiae* and focus on the role of the *mutL* homolog *MLH1* (Prolla et al. 1994a). In mouse, Mlh1 is required for normal meiotic progression (Baker et al. 1996; Edelmann et al. 1996). In male *mlh1* -/- mice, although chromosome synapsis appears normal, pachytene arrest and subsequent apoptosis lead to sterility. Immunolocalization of the Mlh1 protein in normal mice reveals a punctate pattern of staining along the lengths of pachytene chromosomes (Baker et al. 1996). The observations that some foci mark the sites of chiasmata (the cytologically visible manifestation of crossing-over) and that chiasmata are reduced 10- to 100-fold in Mlh1-deficient mice is suggestive of a role for Mlh1 in the process of crossing-over. Meiosis is thought to be disrupted at a similar stage in female *mlh1* -/- mice, which are also sterile (Edelmann et al. 1996).

We show that Mlh1-deficient yeast have reduced crossing-over, increased non-Mendelian segregation, and high frequencies of postmeiotic segregation (PMS). These results demonstrate that a MutL homolog is required for both crossover and gene conversion pathways of meiotic recombination.

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Results

Frequencies of crossing-over in mismatch repair mutants

Map distances were calculated from tetrad data for four or five genetic intervals in wild type and *mlh1*, *pms1*, *msh2*, *msh3*, and *msh6* mismatch repair mutant backgrounds (Table 1). In *mlh1* mutants, map distance is reduced significantly in all five intervals ($P < 0.05$ to $<< 0.001$), with an overall reduction of 33% for the combined genetic distance. In addition, *mlh1 msh2* double mutants are indistinguishable from the *mlh1* single mutants with respect to crossing-over (Table 1). The one exception is the *HIS4–CENIII* interval, which has significantly less crossing-over in the *mlh1 msh2* double mutant than in the *mlh1* single mutant ($P < 0.05$). A reduced frequency of crossing-over is not observed in any of the other mismatch repair mutants examined.

Small but significant increases in map distance are observed in some intervals for the other mismatch repair mutants (see Table 1, footnote). Most notable is the interval *TRP5–CENVII*, which is expanded by 41% and 33% in *msh2* and *msh3* mutants, respectively ($P < 0.001$). It should be noted that, although all diploids used in this study are isogenic, the two haploid strains that constitute every diploid are congenic with each other. The increases in map distance may be a reflection of this congenicity (see Discussion section).

Genetic epistasis of MLH1 *with* MSH4

The crossover deficit of *mlh1* is similar to that observed in yeast *msh4* and *msh5* mutants (Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995). This prompted us to investigate the relationship between the *MLH1* and *MSH4* genes. Because of the extremely poor spore viability in *msh4* mutants (Fig. 1; see Table 3 below; Ross-Macdonald and Roeder 1994), random spore analysis was used to examine recombination in the two single mutants and the *mlh1 msh4* double mutant. These studies

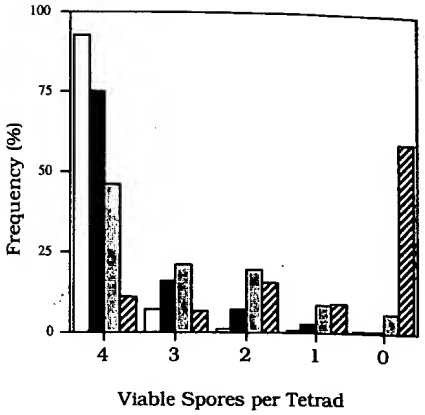

Figure 1. Patterns of spore viability for wild-type (open bar), *msh2* (solid bar), *mlh1* (shaded bar), and *msh4* (hatched bar) strains. The distribution of tetrads with four, three, two, one, and zero viable spores are shown. Between 375 and 638 tetrads were dissected for each strain. *mlh1* and *msh2* strains were sporulated under conditions that prevent vegetative growth as a diploid (see text for discussion).

suggest that an epistatic relationship exists between *MLH1* and *MSH4* (Table 2). As expected, all three strains have reduced frequencies of recombination relative to wild type ($P < 0.001$). Also, nonmating colonies, indicating nondisjunction of chromosome III, are observed in these mutant strains. The *msh4* single mutant has a more severe phenotype than *mlh1*, having 1.4-fold less recombination and five times as many nonmating colonies ($P < 0.05$). However, the *mlh1 msh4* double mutant is indistinguishable from the *msh4* single mutant ($P > 0.8$), a result not expected if the two genes were acting independently to promote crossing-over. The size of the data set presented in Table 2 is sufficient to detect an additive effect of these mutants.

Spore viability in mismatch repair mutants

Several studies have shown that mismatch repair mu-

Table 1. *Reciprocal exchange*

	Interval																
	HIS4–CENIII			*TRP5–CENVII*			*CAN1–CENV*			*HIS4–MAT*				*MAT–URA3–MAT*			
Genotype	T	N + P	cM	T	N + P	cM	T	N + P	cM	T	N	P	cM	T	N	P	cM
Wild type	1441	1173	27.6	913	2046	15.4	1635	611	36.4	517	60	244	53.4	204	5	638	13.8
mlh1	386	599	19.6	164	454	13.3	590	586	25.1	313	25	346	33.8	18	1	176	6.1
pms1	381	260	29.7	269	507	17.3	497	195	35.9	———	N.D.	———		28	0	120	11.7
msh2	484	322	30.0	406	530	21.7	592	268	34.4	185	15	71	50.7	42	2	153	13.7
msh3	380	365	25.5	367	528	20.5	644	242	36.3	———	N.D.	———		58	2	233	11.9
msh6	336	258	28.3	216	421	16.9	444	184	35.3	———	N.D.	———		26	0	115	9.2
mlh1 msh2	99	207	16.2	83	288	11.2	163	202	22.3	63	4	87	28.2	26	1	170	8.1

(T) Tetratype; (N) nonparental ditype; (P) parental ditype, (cM) centiMorgans; (N.D.) not determined. Tetrads were analyzed for reciprocal exchange in the five intervals shown. Map distance in cM was calculated using second division segregation for marker to centromere (*ade1/ADE1*) distances or according to Perkins (1949) for intergenic distances. For the *HIS4–CENIII* and *TRP5–CENVII* intervals the *pms1*, *msh2*, and *msh3* data sets differ from wild-type ($P < 0.05$ to < 0.001). Additionally, the *msh2* mutant has significantly less exchange than wild type in the *CAN1–CENV* interval ($P < 0.05$). (See text for further details.)

Table 2. *Analysis of random spores*

Relevant genotype	Percentage recombinants[a]		Percent nonmating colonies[b]
	HIS4–MAT	*TRP5–CYH2*	
Wild type	40 (81/200)	44 (89/200)	0.0 (0/200)
mlh1	26 (73/280)	22 (62/280)	1.1 (3/280)
msh4	20 (41/200)	13 (27/200)	5.5 (11/200)
mlh1 msh4	20 (49/240)	14 (34/240)	5.4 (13/240)

[a]For random spores, the map distance in cM is approximately equivalent to the frequency of recombinants. The *mlh1* single mutant differs from both *msh4* and *mlh1 msh4* in the *TRP5–CYH2* interval ($P < 0.05$) but not at *HIS4–MAT*. With the size of the current data set the frequency of nonmating haploids produced by the *mlh1* diploid is not different from the wild-type strain ($P = 0.08$). (See text for details.)

[b]Nonmating colonies represent nondisjunction of chromosome III. A nondisjunction rate of 1.05×10^{-3} has been determined for wild-type strains (Goldway et al. 1993).

tants suffer from significant reductions in spore viability, which has been attributed to their mutator phenotypes (Williamson et al. 1985; Kramer et al. 1989; Reenan and Kolodner 1992b; New et al. 1993; Prolla et al. 1994a). Spore viabilities for wild-type and mismatch repair mutant diploids are shown in Table 3. Our standard sporulation media contains a small amount of glucose, which allows diploids to complete a final mitotic division before meiosis, thereby improving the efficiency of sporulation (see Materials and Methods). When *mlh1*, *pms1*, *msh2*, and *msh3* diploids are sporulated under these conditions the *mlh1* strains have 16% fewer viable spores than the least viable of the other three mutant strains, *pms1* ($P < 0.001$). *pms1* and *mlh1* mutants have indistinguishable mutator phenotypes (Prolla et al. 1994a), suggesting that the additional 16% spore death is not exclusively attributable to the accumulation of haplolethal mutations during vegetative growth as a diploid. Sporulation on media without glucose prevents any mitotic growth as a diploid and alleviates 2.5% spore death in a *msh2* diploid and 12% spore death in a *mlh1* strain. The same sporulation regime results in essentially wild-type spore viability for *msh6* strains. The data for *mlh1* and *msh2* mutants are compared to both wild-type and *msh4* strains in Figure 1. Wild-type diploids are highly viable, with a random distribution of dead spores. All mutants have a highly nonrandom pattern of spore death ($P << 0.005$). The spectra of spore death are similar between *mlh1* and *msh2*, except that *mlh1* strains produce 7% fewer viable spores (1609 of 1920 and 1359 of 1500; $P << 0.001$). The additional spore death of *mlh1* mutants appears to be confined to tetrads with two and zero viable spores (*mlh1*, 68 of 480 tetrads had two or zero viable spores; *msh2*, 26 of 375; $P < 0.001$). Tetrads with two and zero viable spores are also the major classes of tetrads in *msh4* mutants (Fig. 1; Ross-Macdonald and Roeder 1994). These classes of tetrad are expected when one or more pairs of homologous chromosome missegregate at the first meiotic division. The results from random spore analysis suggest an increase in nondisjunction of chromosomes in *mlh1* mutants (see above; Table 2). Meiosis I nondisjunction was assessed further by both genetic and physical methods (see Materials and Methods). Of 480 tetrads from a *mlh1* diploid, 46 produced two viable spores. Three of these formed two nonmating colonies, suggesting disomy for chromosome III and corresponding to a nondisjunction rate of $\sim 0.6 \times 10^{-2}$ per meiosis. This is consistent with the data from analysis of random spores (Table 2). Chromosome nondisjunction in wild-type cells occurs at a rate of $\sim 1.4 \times 10^{-4}$ to 1.05×10^{-3} per meiosis (Goldway et al. 1993; F.E. Pryde and E.J. Louis, pers. comm.), but was not determined in the strain background used in this study. The chromosome III disomes were confirmed physically when 41 of the 46 tetrads with two viable spores were karyotyped successfully by contour-clamped homogenous electric field (CHEF) gel analysis (Hunter et al. 1996). Nondisjunctions of chromosomes I and X were also detected (three and two events, respectively; not shown). Although not a rigorous examination of chromosome missegregation, this analysis suggests that there is an increase in the frequency of meiosis I nondisjunction in *mlh1* mutants.

Elevated frequencies of non-Mendelian segregation and PMS in mlh1 *mutants*

The frequencies of non-Mendelian segregation were examined in wild-type and mutant strains by tetrad dissection. In Table 4 tetrad data for three defined alleles of the *HIS4* gene are presented. In *mlh1* mutants, the frequencies of non-Mendelian segregation at these three alleles

Table 3. *Spore viability*

Genotype (strain)	Percentage spore viability
Wild type	97.69 (2493/2552)
mlh1[a]	83.80 (1609/1920)
mlh1	71.16 (2354/3308)
pms1	87.12 (3349/3844)
msh2[a]	90.60 (1359/1500)
msh2	88.13 (4262/4836)
msh3	90.93 (2506/2756)
msh4	25.30 (511/2020)
msh6[a]	96.68 (1543/1596)

Diploids were sporulated and tetrads dissected.

[a]Diploids were sporulated under conditions that prevented any growth as a diploid.

Table 4. *Non-Mendelian segregation at* HIS4

his4 allele	Genotype (strain)	Tetrad class 6:2	2:6	5:3	3:5	Ab4:4	other	Percent total events	Percent PMS/total
his4-Cl	wild type[a,b]	228	258	0	0	0	6, 8:0 13, 0:8	14.9 (505/3390)	0.0 (0/505)
	mlh1[a,b]	7	12	76	95	15	1, 7:1 1, 1:7 1, D5:3 1, D3:5	21.0 (209/997)	90.9 (190/209)
	pms1[a,b]	8	29	33	33	2	2, 0:8 1, Ab6:2	13.2 (108/816)	63.9 (69/108)
	msh2[a,b]	27	19	47	54	2	1, 0:8 1, 1:7	15.3 (151/984)	68.9 (104/151)
	msh3[a,b]	70	54	19	11	1	1, 8:0 1, 0:8 1, 1:7 1, Ab6:2	17.3 (159/919)	20.7 (33/159)
	msh6[a,b]	26	24	1	2	0	1, 0:8	7.9 (53/674)	5.7 (3/53)
	msh2 mlh1[a] (NHD 133)	2	2	16	14	4	1, Ab6:2	18.6 (39/210)	89.7 (35/39)
his4-X	wild type (RHB2588)	15	18	0	0	0	0	11.1 (33/98)	0.0 (0/33)
	mlh1 (NHD128,129)	24	2	24	15	4	0	24.7 (69/279)	62.3 (43/69)
	msh2 (NHD161)	1	2	8	8	1	0	11.7 (20/171)	85.0 (17/20)
	mlh1 msh2 (NHD155)	10	2	17	10	2	1, D5:3	21.6 (42/194)	71.4 (30/42)
his4-Cl	wild type (NHD150)	33	25	0	0	0	1, 8:0 1, 0:8	8.7 (60/689)	0.0 (0/60)
	mlh1 (NHD131,132)	2	4	23	31	4	0	15.9 (65/409)	89.2 (58/65)
	msh2 (NHD162)	4	1	5	3	0	1, 0:8	9.6 (14/145)	57.1 (8/14)
	mlh1 msh2 (RHB2644)	1	1	14	10	3	0	26.6 (29/109)	93.1 (27/29)
his4-RI	wild type (NHD153)	9	10	0	0	0	0	8.3 (19/228)	0.0 (0/19)
	mlh1 (NHD156)	10	2	7	11	3	1, 0:8	16.9 (34/201)	61.7 (21/34)

Tetrads producing four viable spores were analyzed for non-Mendelian segregation of the three alleles of *HIS4*. Segregation patterns were classified according to nomenclature taken from eight spored fungi. Sectored colonies (PMSs) were detected using the plate dissection method (Materials and Methods; Fogel et al. 1979). 5:3 and 3:5 tetrads have a single His^+/His^- colony; aberrant 4:4 (Ab4:4), aberrant 6:2 (Ab6:2), and aberrant 2:6 (Ab2:6) have two sectored colonies; deviant 5:3 (D5:3) and deviant 3:5 (D3:5) have three sectored colonies.

[a]These diploids contain the *MAT–URA3–MAT* interval on chromosome III. The *MAT–URA3–MAT* interval comprises a 9-kb, nontandem duplication of the mating-type locus flanking pBR322 and *URA3* sequences (Borts and Haber 1987), which undergoes high frequencies of crossing-over during meiosis (Table 1). Interestingly, in wild-type strains, the frequency of non-Mendelian segregation at *his4-Cl* is increased 1.7-fold by the presence of this structure ($P << 0.001$), although the two loci are located some 132 kb away, on opposite sides of the centromere. The *mlh1* diploids have a higher frequency of non-Mendelian segregation than all other equivalent ($P < 0.05$ to $<< 0.001$), excluding the *msh2 mlh1* double mutant strains (NHD133 and NHD155). The proportion of events displaying PMS at *his4-Cl* is significantly greater in *mlh1* strains than in the equivalent *pms1*, *msh2*, *msh3*, and *msh6* strains ($P << 0.001$). (Also see text and Fig. 2.)

[b]These data sets represent pools of diploids that contain different versions of the *MAT–URA3–MAT* interval but are otherwise isogenic.

are elevated 1.4- to 2.2-fold above wild type ($P < 0.001$; Table 4; Fig. 2). In contrast, the total frequencies of non-Mendelian events at the *his4-X* and *his4-Cl* alleles are not increased significantly by mutation of *MSH2* (NHD161 and NHD162; *his4-RI* was not tested). Also, with the exception of *msh6* (see below), the other mismatch repair mutants do not alter significantly the total frequency of events at *his4-Cl*. In wild-type cells the

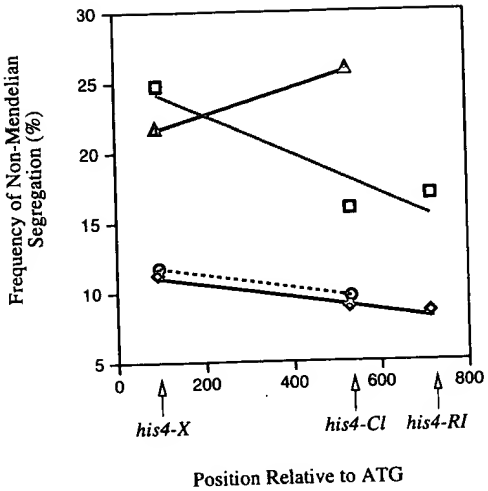

Figure 2. Frequency of non-Mendelian segregation at *HIS4*. The total frequencies of non-Mendelian segregation for *his4-X* (at position +96 in the *HIS4*-coding region), *his4-Cl* (+533), and *his4-RI* (+718) for wild-type and *mlh1* strains are shown (Table 4). Data for *his4-X* and *his4-Cl* in *msh2* single mutants and *mlh1 msh2* double mutants are also plotted. None of these strains contain the *MAT–URA3–MAT* interval. A 1.8- to 2.2-fold increase in the frequencies of events is observed in *mlh1* strains (□), relative to wild type ($P < 0.01$ to $<< 0.001$) (◇). In contrast, *msh2* (○) strains do not have significantly elevated frequencies of aberrant segregation at *HIS4* ($P > 0.7$). In a *mlh1 msh2* double mutant (△), the frequency of events at *his4-Cl* is increased relative to the *mlh1* single mutant ($P < 0.05$); this is not true at *his4-X*.

his4-X, *his4-Cl*, and *his4-RI* alleles were never observed to undergo PMS and therefore, represent efficiently repaired mismatches. Tetrads with one or more colonies displaying PMS account for 62–91% of all non-Mendelian segregations in *mlh1* mutants. At *his4-Cl* the proportion of events displaying PMS is significantly greater for *mlh1* strains than for the other mismatch repair mutants ($P << 0.001$). At the *his4-X* allele, the *msh2* mutation does not increase the total frequency of non-Mendelian segregation (unlike *mlh1*), but a significantly greater proportion of these events are PMS when compared to *his4-X* in *mlh1* strains (17 of 20 and 43 of 69, respectively; $P < 0.05$).

We also noted that the *his4-Cl* allele (a 2-bp duplication) is repaired nearly four times less frequently than either the *his4-X* or *his4-RI* alleles (4-bp duplications) in *mlh1* strains. The latter also show disparities in the direction of gene conversion, with a 5- to 12-fold bias toward 6:2 events ($P < 0.01$ to < 0.001), indicating preferential excision of the mutant allele.

The HIS4 *gene conversion polarity gradient*

Studies by other investigators have demonstrated that in wild-type cells the *HIS4* gene displays a 5′ to 3′ gradient in the frequency of non-Mendelian segregation of well-repaired alleles (White et al. 1992; Alani et al. 1994). This gradient is disrupted in *msh2* mutants (Reenan and Kolodner 1992b; Alani et al. 1994) and when palindromic loop mismatches (that frequently escape mismatch repair) are examined (Detloff et al. 1992; White et al. 1992). In a strain background that has extremely high frequencies of aberrant segregation at *HIS4*, an approximately twofold gradient is observed over the entire gene. The gradient is just 1.5-fold over the region we have examined (Detloff et al. 1992; White et al. 1992). Given such a shallow gradient and the fact that the wild-type strains used in this study have relatively low frequencies of aberrant segregation at *HIS4*, we could not expect to see a statistically significant polarity gradient unless the data sets were extremely large. However, the gradient of aberrant events between *his4-X* and *his4-RI* is significant in *mlh1* strains ($P < 0.05$; Table 4; Fig. 2). This suggests that mutation of *MLH1* does not disrupt the gene conversion polarity gradient at *HIS4*. Analysis of *mlh1 msh2* double mutants supports this proposal. At *his4-X*, the frequency of non-Mendelian events in a *mlh1 msh2* double mutant (Table 4; strain NHD155) is not significantly different to the equivalent *mlh1* single mutant. However, at *his4-Cl* (Table 4; strain RHB2644), the frequency of events in the double mutant is 1.7-fold higher than in a *mlh1* single mutant ($P < 0.01$), demonstrating the dependence of the *HIS4* polarity gradient on *MSH2* function.

Discussion

A unique role for Mlh1 during meiosis

Among the five MutL and MutS homologs with known mismatch repair activities (Pms1, Mlh1, Msh2, Msh3, and Msh6) only Mlh1 has a role in promoting crossing-over during meiosis. Although small changes in the frequencies of crossing-over are observed in mismatch repair mutants other than *mlh1*, these are almost exclusively increases in recombination frequency. With respect to Pms1 and Msh2 in particular, this is consistent with the antirecombination activity that has been demonstrated for these proteins (Selva et al. 1995; Chambers et al. 1996; Datta et al. 1996; Hunter et al. 1996; N. Hunter and R. H. Borts, unpubl.). This activity functions to prevent genetic exchange between diverged DNA sequences. In yeast diploids with a truly isogenic background (i.e., perfectly homologous chromosomes), *pms1* and *msh2* mutants do not have significantly altered frequencies of exchange (Hunter et al. 1996). Therefore, it seems likely that the small increases in recombination observed for *pms1* and *msh2* are attributable to the low levels of sequence divergence present between the congenic parental haploid strains and the absence of an antirecombination activity.

There are two other prominent features of the meiotic phenotype of *mlh1* mutants: (1) a general increase in the frequency of non-Mendelian segregation and (2) a very high incidence of PMS events.

The crossover function of Mlh1

The crossover deficit observed in *mlh1* diploids is simi-

lar to that of *msh4* and *msh5* yeast mutants. A 1.4- to 3.4-fold and a 1.9- to 4.0-fold reduction in crossing-over are observed in *msh4* and *msh5* strains, respectively (Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995). Studies of the double mutant indicate that the Msh4 and Msh5 proteins function in the same epistasis group to promote crossing-over (Hollingsworth et al. 1995). This process is independent of the presence of mismatches and Msh4 and Msh5 are not involved in mismatch repair. Mlh1 also appears to promote crossing-over independently of heterologies. Although the haploid strain backgrounds used in this study are congenic (and, therefore, may be very slightly diverged), the artificial *MAT–URA3–MAT* intervals are perfectly homologous and display the greatest reduction in exchange (2.3-fold) of all the intervals examined in *mlh1* mutants. Also, if the crossover function of Mlh1 was dependent on the recognition of mismatches, lack of the major mismatch-binding protein Msh2 might be expected to affect the *mlh1* crossover phenotype. The data for a *mlh1 msh2* double mutant indicate that the crossover function of Mlh1 is not Msh2 dependent. Analysis of a *mlh1 msh4* double mutant suggests that the Mlh1 and Msh4 (and presumably Msh5) proteins function in the same pathway to promote reciprocal exchange. We note that *msh4* mutants and the *mlh1 msh4* double mutant have a generally more severe defect with less crossing-over, more chromosome nondisjunction, and lower spore viability than *mlh1* mutants (this study; Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995). Therefore, we tentatively propose that Mlh1 is required at a step after the action of Msh4 and Msh5.

The gene conversion function of Mlh1

Our results indicate that most meiotic gene conversions proceed through a heteroduplex intermediate and the Msh/Mlh system is the major heteroduplex repair pathway. Mlh1 appears to be central to this pathway.

The Pms1 and Mlh1 proteins are believed to function as a heterodimer during mismatch correction (Prolla et al. 1994b; Li and Modrich 1995). Previous genetic data suggested an equal requirement for both gene products in yeast meiosis (Prolla et al. 1994a). However, the crossover function is unique to Mlh1. This indicates that the Pms1/Mlh1 heterodimer is not the exclusive functional form of these proteins. In addition, our observations indicate a greater role for Mlh1 in at least some heteroduplex correction reactions. The 2-bp duplication mismatch created at the *his4-Cl* allele is repaired almost exclusively by a Mlh1-dependent pathway. In contrast, ~38% of heteroduplexes created at the *his4-X* and *his4-RI* alleles (4-bp duplications) are repaired by a Mlh1-independent mechanism. This pathway produces a 5- to 12-fold bias toward removal of the mutant allele, indicating preferential excision of the longer, mutant strand. The formation of heterodimers of Pms1 or Mlh1, with one of the two uncharacterized *mutL* homologs *MLH2* and *MLH3* (Prolla et al. 1994a; *Saccharomyces* genome database), could account for this pathway and explain the phenotypic differences between *pms1* and *mlh1* mutants.

Another unique feature of *mlh1* mutants is the increase in total non-Mendelian segregation. A number of possible explanations can account for this phenotype. First, absence of Mlh1 could lead to an increase in the number of recombinational interactions that are initiated. This proposal predicts a role for Mlh1 at an early stage in the recombination pathway, and would represent a novel activity for MutL proteins. Second, the additional PMS events detected in *mlh1* mutants may represent mismatches that are normally repaired to restore the parental ratio of alleles (4:4 segregation). However, if all mismatch correction during meiosis (conversions and restorations) were dependent on mismatch recognition by the Msh2 protein, then the frequency of aberrant events in *msh2* strains should be equivalent to, or greater than, that observed for *mlh1* and other mismatch repair mutants. The examination of alleles of the *HIS4* gene in this study indicates that this is not the case. About half of the events observed in *mlh1* strains occur independently of the other mismatch repair proteins. Therefore, if frequent restoration does occur in wild-type cells, it is not Msh2 dependent. Moreover, disruption of restorational repair might be expected to disrupt gene conversion polarity (see below). These caveats make the restoration hypothesis less attractive to us. A third possibility is that Mlh1 promotes the formation of a class of recombination intermediates that have short heteroduplex tracts, perhaps by restricting DNA synthesis or eliminating the products of over-replication (see model in Fig. 3 and below). In *mlh1* mutants these events may mature by a different pathway producing intermediates with longer heteroduplexes that are generally resolved as noncrossovers. Alleles will have a greater chance of being incorporated into these longer heteroduplex tracts and an apparently new set of non-Mendelian events will result. Finally, two studies have demonstrated that non-Mendelian segregation is asymmetric with respect to recombination initiation sites (i.e., non-Mendelian segregation occurs on only one side of the initiation site; Porter et al. 1993; Gilbertson and Stahl 1996). If Mlh1 were required to generate this asymmetry, an elevated frequency of non-Mendelian segregations might result in *mlh1* mutants. These last two explanations are not mutually exclusive as both result in the formation of more heteroduplex.

Interestingly, small increases in non-Mendelian segregation are also observed in *msh4* and *msh5* mutants yet, unlike *mlh1* mutants, they are not mismatch repair defective. In the study of Ross-Macdonald and Roeder (1994), non-Mendelian segregation was increased by 1.4- to 2.3-fold at four of the six alleles examined. Similarly, Hollingsworth et al. (1995) observed 1.3- to 6.1-fold increases at the three loci they examined. However, given the size of the data sets presented, a significant increase was only recorded for a single locus in each of these studies. In this respect the phenotypes of *msh4* and *msh5* mutants may represent a moderate *mlh1*-like defect in a mismatch repair proficient context.

The *HIS4* gene conversion polarity gradient is not

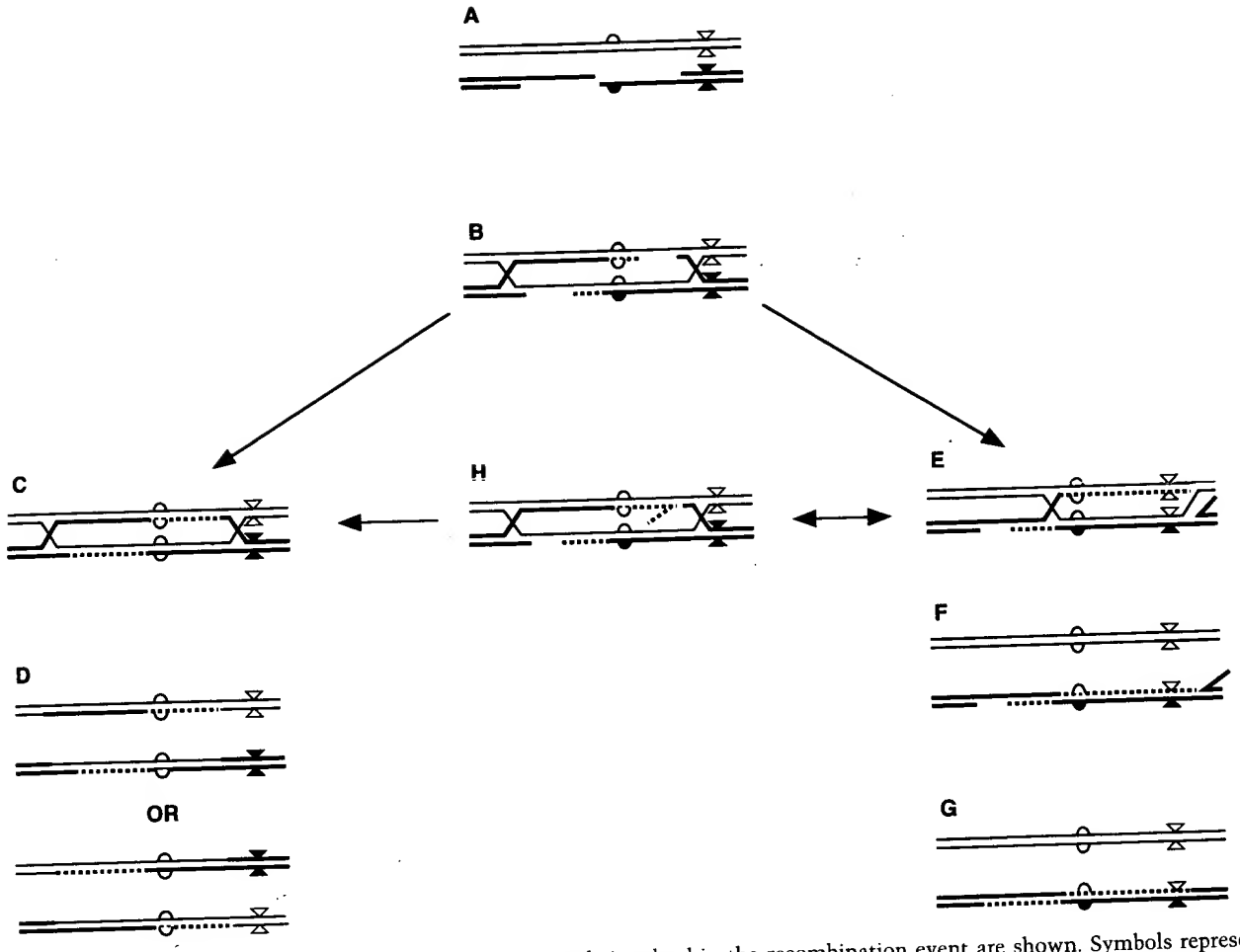

Figure 3. Model for Mlh1 function. Only the two chromatids involved in the recombination event are shown. Symbols represent heterozygous alleles. (*A*) Double-strand cleavage and 5′ to 3′ exonucleolytic resection produces long 3′ single-stranded tails. (*B*) Strand invasion primes DNA synthesis, which displaces the nontemplate strand, allowing the formation of heteroduplex DNA. The crossed strand junctions are bound and stabilized by Msh4 and Msh5. Mlh1 may also promote this transition. (*C*) In wild-type cells heteroduplex DNA is repaired to produce a gene conversion. A covalently closed double Holliday junction is then formed. Although these structures are likely to be the precursors of crossovers (Schwacha and Kleckner 1995) they are not essential for the formation of noncrossovers (gene conversions without an associated crossover; Resnick 1976; Hastings 1988; Schwacha and Kleckner 1995). Noncrossovers may bypass the double Holliday junction stage by unwinding the structure shown in *B*. (*D*) The double Holliday junction is resolved to give crossover or noncrossover recombinants. (*E*) In the absence of Mlh1, Msh4, or Msh5 instability of one or both junctions may lead to over-replication of recombination intermediates resulting in longer heteroduplex DNA. If such an intermediate is going to produce a crossover the cross-junctions must be reestablished to allow double Holliday junction formation (*E*,*H*,*C*). We propose that Mlh1 is required to prevent over-replication or to trim the products of over-replication (shown in *H*). (*F*) In *mlh1* mutant cells recombination intermediates cannot progress efficiently to the double Holliday junction stage and, therefore, the frequency of crossing-over is reduced. The structure is broken down by the action of a helicase, topoisomerase, or junction-specific nuclease. One possible outcome is shown. (*G*) Primarily noncrossover products are produced. Because Mlh1 is required for mismatch correction, heteroduplex is not repaired resulting in PMS. Note that the heteroduplex tract is longer than the intermediate shown in *B*. (See text for further discussion.)

abolished in *mlh1* mutants. However, the gradient is disrupted by mutation of *MSH2*, even in the absence of *MLH1* function. As *mlh1* mutants are severely repair deficient, this observation suggests that the *HIS4* polarity gradient may depend on the recognition, but not the correction, of mismatches. This interpretation supports the proposal that Msh2 blocks the extension of heteroduplex when a mismatch is encountered (Alani et al. 1994). An alternative explanation of polarity is that a gradient in the direction of mismatch correction extends from an initiation site for heteroduplex formation (Detloff et al. 1992). In this model, repair toward the conversion of an allele predominates close to the initiation site, whereas restorational repair is more frequent further along a heteroduplex tract. In the light of the data presented for *mlh1* mutants this model is difficult to accommodate as the *HIS4* polarity gradient occurs in the absence of mismatch correction.

Models for Mlh1 function

Ross-Macdonald and Roeder (1994) have suggested that the Msh4 protein may promote reciprocal exchange by stabilizing junction structures, thereby counteracting the forces that could destroy early recombination intermediates. This stabilization activity may be a common feature of several MutS homologs. For example, the Msh2 and Msh3 proteins are components of the Rad1/10 pathway of mitotic recombination (Saparbaev et al. 1996). More specifically, Msh2 and Msh3 are required for single-strand annealing reactions (N. Sugawara, F. Pâcques, M. Colaiácovo, and J.E. Haber, pers. comm.) where they may stabilize the junctions between double-stranded and 3′ single-stranded tails and then recruit the Rad1/10 endonuclease to cleave the 3′ tails (Ivanov and Haber 1995). The *S. pombe* MutS homolog Swi4 may also be involved in junction recognition (see below). In the context of a stabilization mechanism, we propose that Mlh1 acts after Msh4 and Msh5 to reinforce the stabilization and promote progression to crossover resolution. The fact that Mlh1 does not appear to be required for mitotic recombination (Saparbaev et al. 1996) indicates that this is a meiosis–specific specialization.

To account for longer heteroduplex in *mlh1* mutants we propose that Mlh1 is required to prevent the over-replication of recombination intermediates or to trim the products of over-replication, thereby allowing the formation of a double Holliday junction (see Fig. 3). Interaction with an endonuclease may be required to execute this step. This may be analogous to the way that MutL is thought to function as an interface between the mismatch binding and excision and resynthesis reactions of mismatch repair (for review, see Friedberg et al. 1995). The *S. pombe* MutS homolog Swi4 may be a component of a similar pathway during mating-type switching. In *swi4* mutants copying of the storage cassettes continues unchecked, resulting in duplications of the *mat* region (Fleck et al. 1992). It has been proposed that the binding of Swi4 to some secondary structure that resembles a mismatch (or a double-strand/single-strand junction as discussed above) may signal the termination of DNA synthesis. It will be interesting to see whether this pathway also requires the activity of a MutL homolog.

Comparison of the yeast and mouse mutants (Baker et al. 1996; Edelmann et al. 1996) suggests that the meiotic function of Mlh1 may be conserved throughout eukaryotes. In yeast *mlh1* mutants, meiosis proceeds with substantially normal kinetics (N. Hunter and R.H. Borts, unpubl.). In male *mlh1* −/− mice, meiosis arrests at pachytene with fully synapsed autosomes but separated X and Y chromosomes. With ~25 chiasmata per nucleus and 20 chromosome pairs (Lawrie et al. 1995), a reduction in crossing-over of 1.5-fold (as in *mlh1* yeast) would result in frequent achiasmate chromosomes in *mlh1* −/− mouse meiosis. In fact, chiasma appear to be reduced by 10- to 100-fold in *mlh1* −/− mice (Baker et al. 1996). The presence of multiple achiasmate bivalents, particularly the sex chromosomes (Burgoyne et al. 1992), may trigger cell-cycle arrest and subsequent apoptosis. Therefore, we believe that the ability of *mlh1* yeast to progress through meiosis does not reflect a difference in the functions of yeast and mouse Mlh1 proteins, but is attributable to the different physiology of yeast and mouse meioses.

Materials and methods

Plasmids

The *LEU2* gene was inserted into a *Sna*BI site in the wild-type *MSH2* gene contained in plasmid pII-2 (Reenan and Kolodner 1992a) to produce pRHB113 (*msh2::LEU2*). PWK4–*pms1* is a deletion of almost the entire *PMS1* open reading frame (ORF) (Kramer et al. 1989). The deletion was marked by inserting the *LEU2* gene into an adjacent *Xba*I site to create the plasmid pRHB197. p*mlh1ΔLEU2* is a 530-bp deletion of the 5′ coding region and upstream sequences of *MLH1* marked with the *LEU2* gene (Prolla et al. 1994a) and was kindly provided by Dr M. Liskay (Oregon Health Sciences University, Portland). pGEM7Zf(+)/ΔCH*LEU2* is a disruption/deletion of *MSH3* created by replacing a *Cla*I–*Hpa*I fragment of *MSH3* with the *LEU2* gene (a kind gift of G. Carignani, Università degli Studi di Padova, Italy). A 4-kb PCR fragment containing the *MSH6* gene was cloned into the *Srf*I site of pPCRScript (Stratagene). A *Pvu*II–*Eco*RV fragment containing the kanMX4 module (Wach et al. 1994) was then used to replace a *Sna*BI–*Spe*I fragment of the *MSH6* ORF to produce the plasmid pSRC9 (*msh6Δ::*kanMX4; kindly provided by S.R. Chambers, Oxford University, UK). pRHB11, pRHB12, and pRHB13 contain *Xho*I, *Eco*RI, and *Cla*I restriction site fill-in mutations of the *HIS4* gene.

Strains

All haploid strains are isogenic derivatives of either H330 (*ura3-1 can1 ade1 lys2-c met13-2 cyh2 trp5-1 leu2-K MATα*) or RHB2096-1a (*ura3-1 lys2-d met13-4 cyh2 leu2-R MATa*). RHB2096-1a was created by transformation of the *ade1* strain H394 with a 1.4-kb *Xho*I fragment containing the wild-type *ADE1* gene and selecting for Ade$^+$ transformants. H330 and H394 are congenic and have been described previously (Borts and Haber 1989). Although all diploids are derived from this congenic pair of haploid parents they still constitute an isogenic set. The *his4-X*, *his4-Cl*, and *his4-RI* alleles were introduced by a two-step gene replacement. Combinations of the above markers are segregating in all strains except NHD132, which contains *trp1-H3*, a restriction-site fill-in mutation. Mismatch-repair mutants were obtained by one-step gene replacement with the appropriate fragments from the plasmids pRHB113 (*msh2::LEU2*), pRHB197 (*pms1Δ::LEU2*), p*mlh1Δ::LEU2* (Prolla et al. 1994a), pGEM7Zf(+)/ΔCH*LEU2* (*msh3Δ::LEU2*), or pSRC9 (*msh6Δ::*kanMX4). The *msh4* mutation is a complete deletion of the *MSH4*–coding region obtained using PCR-mediated gene disruption (Wach et al. 1994). Mutant strains are referred to by their relevant genotypes (e.g., *mlh1*). Double mutant combinations were obtained by crossing the appropriate isogenic single mutants. The *MAT–URA3–MAT* interval has been described (Borts and Haber 1987) and was introduced by integrative, site-directed transformation (Orr-Weaver et al. 1983). Transformations were verified by Southern blot (Southern 1975; Sambrook et al. 1989) using the digoxigenin, nonradioactive system as recommended by the manufacturer (Boehringer Mannheim).

Genetic procedures

Yeast manipulations and media are as described previously (Rose et al. 1990). Strains were grown at 30°C on YPD medium and synthetic complete media lacking one or more nutritional

supplements. Mismatch repair mutant diploids were made by mixing approximately equal amounts of the parental haploids in 100–200 µl of liquid YPD and then spreading the mixture on solid YPD plates. Mating was allowed to proceed for 6 hr at 30°C or up to 12 hr at room temperature before replicating directly to sporulation medium. Sporulation was performed at room temperature on plates containing 2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 2.5% agar, and 0.09% complete amino acid mixture, or 2% potassium acetate, 2.5% agar, and 0.09% complete amino acid mixture. The latter recipe does not permit vegetative growth and was found to improve the viability of mismatch repair mutant strains. Dissected tetrads were grown for 3–4 days at 30°C. Only spores that formed colonies visible to the naked eye were scored as being viable. Sectored colonies were detected using the plate dissection method (Fogel et al. 1979). Ambiguous sectored colonies were streaked onto YPD plates to obtain single colonies and then replicated to selective media. A mixture of prototrophic and auxotrophic colonies was scored as a PMS. Random spores were prepared as described (Lichten et al. 1987) and grown on synthetic complete medium lacking arginine and containing cycloheximide (10 mg/liter) and canavanine (40 mg/liter) for 3–4 days at 30°C. One-step and two-step gene replacements were performed as described (Rose et al. 1990). Yeast transformation was carried out using a modification of the lithium acetate method (Gietz et al. 1992).

PCR-mediated gene disruption

PCR-mediated disruption of the *MSH4* gene was performed as described (Wach et al. 1994), using the 64-mer deoxyoligonucleotides AGTTATAGCATTGAAATCTGTAGCTGATCAACGCAAACTATATGCACGTACGCTGCAGGTCGAC and CAGAAATAATGGATTATAGTTTTAAGCTAAGCGGCAAAAGCCAAAATCGATGAATTCGAGCTCG and the pFA6–kanMX4 plasmid [containing the *kan*^{r} geneticin resistance gene (Wach et al. 1994)]. Transformants were selected on YPD media containing 400 mg/liter of geneticin (G148, Boehringer Mannheim).

Karyotyping of segregants

Random spore segregants were karyotyped as described (Naumov et al. 1992). Normally, disomy can be assigned accurately by eye for the ten smallest *S. cerevisiae* chromosomes (Hunter et al. 1996), however, the comigration of chromosomes V and VIII in the parental strains RHB2096-1a and H330 made assignment of disomy for these chromosomes difficult, and therefore, only eight chromosomes could be analyzed.

Data analysis

Data sets were analyzed using the standard normal and G-tests as described (Sokal and Rohlf 1969). The G-test is equivalent to the X^2 contingency test. Values of $P < 0.05$ were considered significant.

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